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Modeling Lignification in Grasses with Monolignol Dehydropolymerisate–Cell Wall Complexes

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p-Hydroxycinnamyl alcohols are efficiently polymerized into primary walls from maize cell suspensions by H_2O_2 and wall-bound peroxidases to produce monolignol dehydropolymerisate–cell wall (DHP–CW) complexes. The structure and distribution of the synthetic lignins in complexes are similar to natural lignins formed in grass cell walls. This system was used to elucidate how ferulate-polysaccharide esters act as initiation sites for lignification, cross-link plant cell wall polymers, and restrict enzymatic degradation of structural polysaccharides. In addition, the effects of lignin composition and lignification conditions (pH and rate of precursor addition) on polysaccharide degradability were investigated. DHP–CW complexes are useful for modeling matrix interactions in lignified plant cell walls and for identifying means for improving the utilization of lignocellulosic materials for nutritional and industrial purposes.

Lignification plays an important role in plant growth and development by improving water conduction through xylem tracheary elements, enhancing the strength of fibrous tissues, and limiting the spread of pathogens in plant tissues (*1*). Lignin restricts the degradation of structural polysaccharides by hydrolytic enzymes, thereby limiting the bioconversion of fibrous crops into animal products or into liquid fuels (*2, 3*). Lignified dietary fiber also plays an important role in maintaining gastrointestinal function and health in humans (*4*). The effect of lignin on plant cell wall properties is thought to arise from the hydrophobicity of lignin, and its incrustation and attachment to other matrix components. The extent and importance of these interactions in affecting plant cell wall properties is poorly understood.

Lignification has been modeled *in vitro* through the dehydropolymerisates (DHPs) formed by the oxidative coupling of monolignols (*5-8*). The DHPs formed in the presence of carbohydrates, feruloylated oligosaccharides or proteins have been used to identify potential interactions between lignin and other cell wall components (*9-14*). Unfortunately, DHPs differ structurally from natural plant lignins (*15-17*) and they do not adequately reflect the three-dimensional structure of lignified cell walls. These limitations may be overcome in part by forming DHP–CW complexes using

in situ peroxidases. Whitmore first used this approach in the late 1970's to study lignin-protein and lignin-carbohydrate interactions in cell walls isolated from the callus of *Pinus elliottii* (*18, 19*). Our objective was to further develop, evaluate, and utilize DHP–CW complexes as a means for studying lignin-matrix interactions in grass cell walls and for assessing the effect that such interactions have on the enzymatic degradability of cell walls.

Formation and Evaluation of DHP–CW Complexes

DHP–CW complexes are formed with cell walls isolated from cell suspensions of maize (*Zea mays*, cv. Black Mexican) (*20*). Cell walls from maize cell suspensions and similar tissues (*e.g.* maize coleoptiles) are composed of about 10% protein, 10% pectin, 50% hemicellulose (glucuronoarabinoxylans and heterologously linked glucans), and 25% cellulose (*21-23*). The cell walls also contain about 2% ferulic acid and 0.3% guaiacyl lignin (*20, 21*). Overall, the composition of the cell walls is typical of non-lignified primary walls in grasses.

As with other plant species, maize suspensions secrete peroxidases into the culture medium (Grabber, J.H., unpublished data). If lignin precursors are added to maize suspensions or isolated cells, secreted peroxidases would probably form DHPs in solution (*24*) or on the surfaces of cell walls (*25*) rather than within the wall matrix. Therefore, the cells are ruptured and extracted overnight with 100-200 mM $CaCl_2$ to remove extracellular and loosely bound peroxidases from the walls. However, even after extensive extraction with $CaCl_2$, a small quantity of peroxidase will gradually diffuse into the reaction medium during lignification. Cell walls isolated in this manner contain at least 9 peroxidase isozymes, primarily those of acidic pI (*20*). Multiple peroxidase isozymes are present in cell walls of plants and those of acidic pI are probably involved in lignification (*26*). Peroxidases in walls isolated from maize cell suspensions, like those isolated from maize internodes, will polymerize a variety of monolignols into DHPs, but their substrate affinity is greatest for ferulate and coniferyl alcohol, intermediate for *p*-coumarate, and low for sinapyl alcohol (Hatfield, R.D., unpublished data).

For complex formation, $CaCl_2$ extracted cell walls are thoroughly washed with water and suspended in 10-50 mM buffer solutions adjusted to pHs between 3.5 and 7.5 (*20*). Complexes may be formed in citrate, phosphate, HOMOPIPES or PIPES buffers. We generally use PIPES or HOMOPIPES buffers because they are not oxidized by hydrogen peroxide, they do not cause precipitation of calcium (needed for peroxidase activity and for maintaining the structural integrity of ionically bound pectins) and they do not form stable addition products with the quinone methide intermediates generated during the lignification process. In most cases, cell wall suspensions are lignified by adding a solution of monolignols and glucose over a 24 to 48 h period ('end-wise' polymerization). Monolignols are polymerized into cell walls by wall-bound peroxidases and hydrogen peroxide generated *in vitro* from glucose by glucose oxidase (added to the reaction medium prior to lignification). Since gluconic acid is a coproduct of this reaction, a relatively high buffer concentration (*e.g.* 50 mM) is required to maintain the pH. If desired, a dilute solution of hydrogen peroxide (*e.g.* 0.03%) may be added to the reaction medium in place of *in vitro* generated hydrogen peroxide.

Complexes with a Klason lignin content of up to 20% are readily formed with a variety of monolignols (*20*, Grabber, J.H., unpublished data). The maximum Klason lignin content observed in maize internodes is also about 20%. Incorporation of monolignols into cell wall-bound DHPs by 'end-wise' polymerization is usually over 90%. Yields are reduced either if the monolignol solutions include a high proportion of sinapyl alcohol (>60 mole %), or if hydrogen peroxide is added rapidly (< 1 h) to cell walls suspended in monolignol solutions ('bulk' polymerization), or if 'end-wise' polymerization is carried out under relatively acidic conditions (pH <4.5). Only in the latter case are the low yields attributable to peroxidase inactivation. The low yields of syringyl DHP–CW complexes are probably due to a low affinity on the part

of the peroxidases for sinapyl alcohol. In the case of 'bulk' polymerization, DHPs are readily formed, but only about one-half is bound to the cell walls.

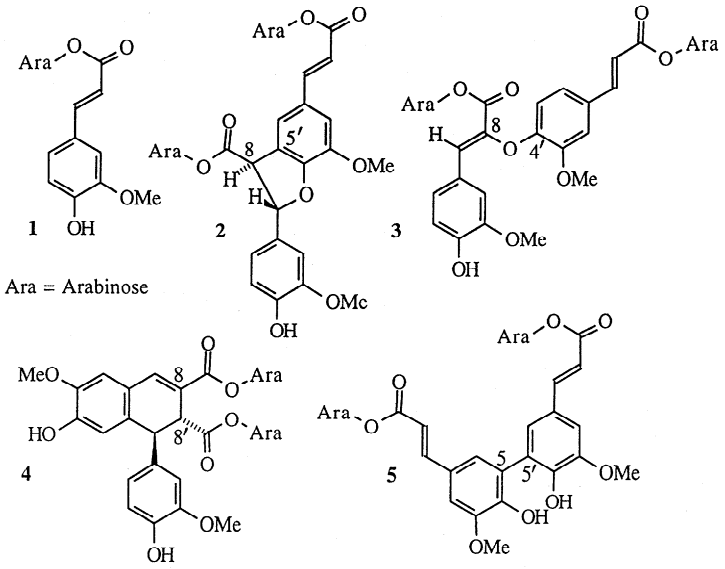
Thioacidolysis GC-MS, pyrolysis GC-MS and proton-decoupled ^{13}C-NMR analyses of the complexes and lignins isolated indicate that the synthetic lignins formed in DHP–CW complexes are structurally similar to natural lignins formed in grasses. In addition the DHPs, like natural grass lignins, are distributed throughout the wall matrix (*20*). Although many groups have attempted to produce DHPs which are representative of *in vivo* lignins, our findings suggest that DHP–CW complex formation may be the best system for modeling lignification of grass cell walls.

Applications of DHP–CW Complexes

Elucidation of Matrix Interactions in Cell Walls. Ferulic acid is attached as an ester **1** to the C5-hydroxyl of α-L-arabinosyl moieties of grass arabinoxylans. Recently our group (*27*) demonstrated that oxidative coupling mechanisms result in substantial amounts of 8–5′, 8–*O*–4′, and 8–8′ coupled diferulate esters (**2-4**) in addition to the previously reported 5–5′ coupled product **5**. We used nonlignified walls from maize cell suspensions and DHP–CW complexes to investigate cross-linked structures formed by ferulates in primary cell walls of grasses (*21*, Grabber, J.H., unpublished data). Cell suspensions were grown under normal conditions or with 40 μM 2-aminoindan-2-phosphonic acid (AIP, a selective inhibitor of phenylalanine ammonia lyase) to reduce ferulate deposition into cell walls. When nonlignified cell walls containing normal (17 mg g^{-1}) or low (5.3 mg g^{-1}) ferulate concentrations were incubated with an excess of H_2O_2, 40 to 55% of the ferulate monomers became oxidatively coupled by the wall-bound peroxidases into dehydrodimers. This remarkably high degree of dimerization, even in cell walls with extremely low ferulate concentrations, indicates that the spatial distribution of ferulates is somehow regulated in the maize walls to maximize dehydrodiferulate cross-linking of arabinoxylans. About one-half of the ferulate moieties were coupled by 8–5′ linkages; 8–*O*–4′, 5–5′, and 8–8′ coupled dehydrodimers each comprised 10 to 25% of the total. Ferulate monomers and dehydrodimers differed considerably in their propensity to become incorporated into lignin and to form hydrolyzable ether-linked structures with the lignin (Figure 1). These results indicate that the abundance and importance of ferulates as cross-linking agents has been greatly underestimated by solvolytic analysis of lignified plant tissues.

Oxidative coupling of ferulates to lignin was investigated further by synthetically lignifying cell walls containing γ-^{13}C labeled ferulate (Grabber, J.H.; Ralph, J., unpublished data). GC-MS analysis and long-range C–H correlation NMR spectroscopy studies of alkaline extracts from the complexes revealed that ferulate-coniferyl alcohol cross-products were coupled by 4–*O*–β′, 5–β′ and 8–β′ linkages (**6-8**). Very small amounts of 8–*O*–4′ and 8–5′ coupled products (**9**, **10**) were detected by NMR spectroscopic analysis. In other work, only 4–*O*–β′, 5–β′ and 8–β′ coupled ferulate-lignin structures were detected by NMR spectroscopic analysis of lignins isolated from uniformly ^{13}C-labeled ryegrass (*28*). The virtual absence of 8–*O*–4′ and 8–5′ coupled ferulate-lignin cross-products in these studies indicates that ferulates couple almost exclusively to the β′-position of monolignols; coupling at the 5′ and *O*–4′ positions occurs only with dilignols and lignin oligomers in which the conjugated sidechain is no longer present. The selectivity of the reaction of ferulates with the β′-position in monolignols provides compelling evidence that ferulates act as initiation or nucleation sites for lignification. Similar approaches could be used to investigate the cross-linking of matrix polymers by other cell wall components such as amino acids and uronic acids.

Assessing the Impact of Cell Wall Modifications on Polysaccharide Degradability. Nonlignified cell walls and DHP–CW complexes were used to determine how reductions in ferulate cross-linking affect the degradability of structural polysaccharides by fungal enzymes (Grabber, J.H., unpublished data). In



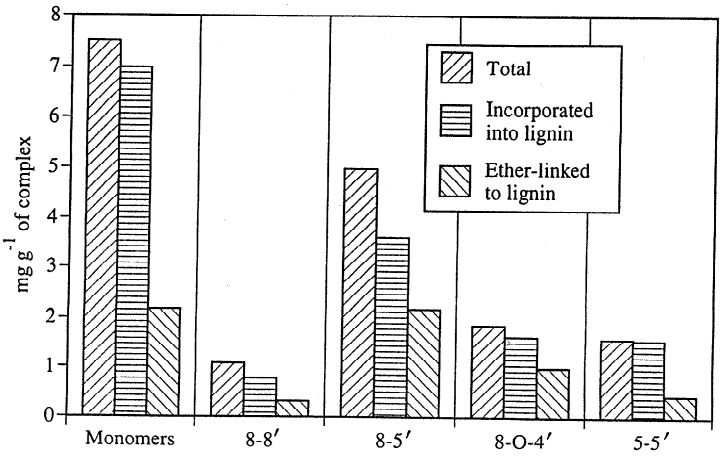


Figure 1. Incorporation of ferulate monomers and dehydrodimers into the lignins formed within DHP–CW complexes.

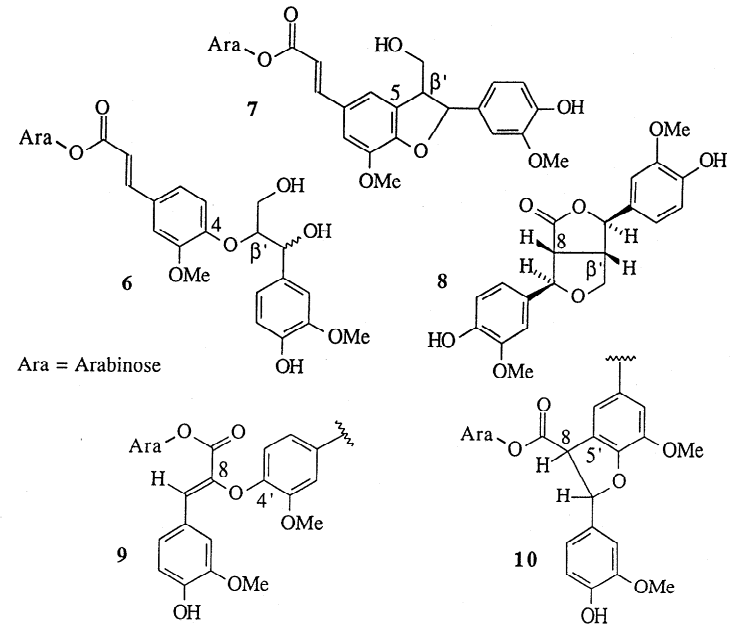
these studies, cell walls from normal or AIP treated cell suspensions were incubated with H_2O_2 or synthetically lignified with coniferyl alcohol/H_2O_2. Reductions in ferulate cross-linking primarily increased the rate of polysaccharide degradation in non-lignified cell walls and the extent of polysaccharide degradation in DHP–CW complexes (Figure 2). Similar results were observed when ferulate cross-linking was reduced by selective methylation of cell wall ferulates with diazomethane prior to complex formation. These results suggest that selection or genetic engineering of grasses for low levels of ferulate cross-linking will significantly improve the degradability of structural polysaccharides. Current studies in our laboratory are focused on determining the extent to which cell wall degradability is restricted by amino acid and uronic acid cross-linking of matrix components.

Lignins are formed by peroxidase/H_2O_2-mediated dehydropolymerization of *p*-coumaryl, coniferyl, and sinapyl alcohols (**11**-**13**). The incorporation of sinapyl alcohol into lignin is reduced in mutant or transgenic plants having low *o*-diphenol *O*-methyltransferase (OMT) activity (*29*). In mutant or transgenic plants where cinnamyl alcohol dehydrogenase (CAD) activity is reduced, *p*-hydroxycinnamyl aldehydes (**14**, **15**) may become major components of the lignin (*30*). DHP–CW complexes were used as a biomimetic system for determining whether changes in lignin composition affect polysaccharide degradability by fungal enzymes (Grabber, J.H., unpublished data). Complexes formed with coniferyl aldehyde were about 40% less degradable than the complexes formed with coniferyl alcohol. Differences in degradability were only partially eliminated if complexes were incubated with high concentrations of fungal enzymes. When aldehyde groups were selectively reduced to alcohols (by ethanolic-sodium borohydride) prior to enzyme hydrolysis, however, degradability differences were eliminated. This suggests that high hydrophobicity of aldehyde-containing lignin might have been responsible for depressed cell wall degradation. Altering the type of *p*-hydroxycinnamyl alcohols used to lignify the walls did not affect subsequent cell wall degradability. Based on these results, we propose that improvements in cell wall degradability, previously attributed to changes in lignin composition (*29, 30*), were in fact due to other modifications in wall chemistry or ultrastructure.

The effect of lignin structure on cell wall degradability was investigated by forming DHP–CW complexes respectively by 'end-wise' and 'bulk' polymerization of coniferyl alcohol (Grabber, J.H., unpublished data). Analysis of the lignin structures by thioacidolysis indicated that DHPs in 'end-wise' complexes had a relatively linear configuration characteristic of natural grass lignins, whereas DHPs in 'bulk' complexes had a highly branched lignin configuration. The degradability of 'end-wise' and 'bulk' DHP–CWs by fungal enzymes was similar. These results indicate that lignin structure does not affect the enzymatic hydrolysis of cell walls.

The pH used to form DHP–CW complexes had considerable impact on polysaccharide degradability, particularly in complexes with high lignin contents (Grabber, J.H., unpublished data). Complexes formed at pH 5.5 were up to 20% more degradable than complexes formed at pH 4.0. The cause of these degradability differences is currently under investigation by our group.

Other Applications. Cell walls from cell suspensions and DHP–CW complexes would be valuable in other areas of research, including investigations on structural-functional relationships of dietary fiber as related to human health. Recently Eastwood and coworkers have used cell walls from cell suspensions of spinach to study the fermentation and metabolism of nonlignified walls by rats (*31, 32*). DHP–CW complexes provide a means of generating lignified fiber with various types of lignin-matrix interactions. Such materials would be valuable in small animal or *in vitro* studies aimed at elucidating the beneficial effects of lignified fiber on gastrointestinal function and health.



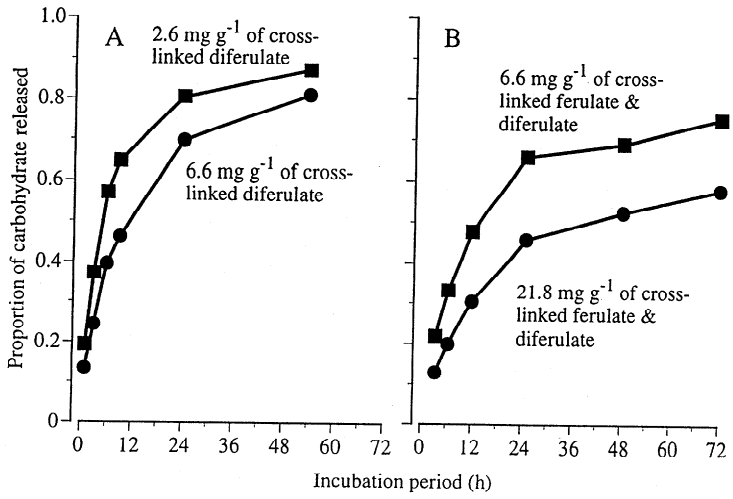
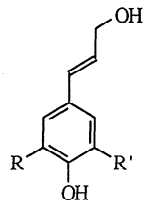
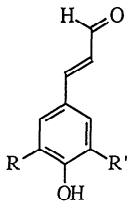


Figure 2. Effect of ferulate cross-linking on the release of carbohydrate from (A) nonlignified walls and (B) DHP–CW complexes during hydrolysis with fungal enzymes.





11; R = R' = H
12; R = H, R' = OMe
13; R = R' = OMe

14; R = H, R' = OMe
15; R = R' = OMe

Limitations of DHP–CW Complexes

Because DHP–CW complexes are formed with primary walls, our system may accurately model lignification of cell types such as parenchyma which have only primary cell walls. Other cell types, like xylem and sclerenchyma, have thick secondary walls in addition to primary walls. Primary and secondary walls both contain noncellulosic polysaccharides, cellulose, structural proteins, hydroxycinnamic acids and lignin (*33-36*), but in differing proportions. Even in these tissues, primary walls are more heavily lignified and more resistant to degradation than secondary walls (*37*) and they limit penetration of degradative organisms and enzymes into the secondary walls (*38*). Since lignification occurs concurrently with secondary wall formation (*33*), it is not possible to adapt our system to cell walls with secondary thickenings because the cell walls must be isolated prior to complex formation.

Secondary cell wall formation and lignification can be stimulated in suspension or callus cultures by the judicious use of phytohormones or other elicitors (*39-42*). Although these systems are valuable for studying the biosynthesis of lignin, the prospect for manipulation of lignin composition or lignin-matrix interactions in these cultures is limited, since it would require reduced expression of a large number of specific enzymes. Developing enzyme inhibitors or genetically altered cultures to manipulate matrix interactions would be time consuming and problematic. As illustrated in the previous examples, DHP–CW complexes give us tremendous flexibility in manipulating and assessing how lignin-matrix interactions affect cell wall properties. Because of the perceived importance of primary cell walls in controlling wall properties, we believe that our system is useful for modeling matrix interactions in lignified walls and for identifying means for improving the utilization of lignocellulosic materials.

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